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Selection of rice aroma from crossing aromatic rice x T250.7 mutant genotype using DNA marker

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Abstract. *Ishak I, Fedora F. 2024. Selection of rice aroma from crossing aromatic rice x T250.7 mutant genotype using DNA marker. Biodiversitas 25: 2191-2197.* Breeding fragrant rice using conventional approach was considered ineffective due to time consuming, therefore molecular breeding using DNA markers was introduced. This research aimed to utilize marker-assisted selection (MAS) for detection of fragrance gene in rice related to molecular breeding. Crossing between aromatic rice (Sinar 1) and non-aromatic rice (T250.7 mutant genotype) showed the results at F1 plants and found the DNA fragment size of 257 bp for fragrance genes in rice was at TS1, TS2, TS3, TS4, TS5, and TS.7 genotypes, whereas, TS6 did not show a fragment DNA at 257 bp. It was predicted that the TS6 genotype did not cross between variety Sinar 1 with T250.7. The F2 seeds were selected and morphologically, they had slender seeds and continued planting for F2 plants. Based on DNA marker identification, it revealed that 75% of the rice plants carried aroma. Similarly, the selected F3 plants continued to be planted and molecular identification indicated that aroma segregation was the same as that in the previous generation. Plants with the number of accessions of 721, 761, and 7210 were predicted to have homogeneity in aroma after detection using molecular markers. The MAS in breeding programs can shorten the duration of rice selection.

Keywords: Aromatic rice, marker-assisted selection, molecular breeding, rice mutant line

INTRODUCTION

The demand for fragrant rice is increasing markedly, it is estimated to account for 15-18% of the rice trade procuring the highest prices on the world market (Giraud 2013). Rice consumers in Southeast Asia prefer the texture and aroma of rice from Thailand, known as jasmine rice. Demand for rice fragrances is largely driven by women, educated consumers, and large families who spend a small portion of their food expenditure on rice (Bairagi et al. 2020). Rice fragrance is a desirable trait and the most important rice quality. The fragrance is attributed to the loss-of-function betaine aldehyde dehydrogenase (BADH2) gene. Identification of allelic variants of BADH2 genes can be applied to rice fragrance breeding programs using traditional molecular markers and Sanger sequencing techniques (Li et al. 2020)

Rice fragrance is caused by the mutation of 8-base pairs of the BADH2 gene acting as suppressor 2-acetyl proline biosynthesis. Mutations in the betaine aldehyde dehydrogenase gene caused free 2-Acetyl proline biosynthesis to produce aroma in rice (Ashokkumar et al. 2020). The proline treatments reduce the transcription level of BADH2 gene activity. In contrast, the grain protein content increases by 3.57-6.51% after treatments using Proline, i.e., Pro2 and Pro3 (Luo et al. 2020). They upregulated the expression of DAO1 during low moisture soil content to promote the conversion from putrescine to 2-AP (Luo et al. 2021).

Marker-assisted selection (MAS) became part of the breeding program routines of important seed companies such as maize, rice, and soybean to accelerate and optimize the cultivar development processes (Sakiyama et al. 2014; Nugraha et al. 2016). Establishing a molecular marker for specific traits can be deployed to identify the results of crossing two varieties. The incorporation of advancement in biotechnology, proteomic research, and combinations of MAS and conventional plant breeding practices create the foundation for molecular plant breeding (Moose and Mumm 2008). Two strategies can be applied to molecular rice breeding programs, such as the use of MAS, also well known as marker-assisted breeding (MAB), and the method of genetically-modified crop development (Wijerathna 2015). In the future, molecular breeding programs will be challenging to make efficient generation of new varieties in breeding (Cobb et al. 2019). The MAS application in plant breeding can accelerate accuracy, continuously empowering plant breeders worldwide (Hasan et al. 2021). The deployment of MAS to generate specific traits is possible when DNA markers are established for these specific traits and selection can commence from F1 plants. According to Fujino et al. (2019), MAS can select gene regions tagged with DNA markers to obtain desirable traits in rice. Marker-assisted backcrossing efficiently selects lines of a target trait and reduces the breeding cycle effectively. The molecular markers can detect the backcross of the BC2F2 lines, which can be used as parental lines for breeding programs purposes with high-quality rice for cooking and meals (Kim et al. 2021).

Fragrance rice with slender seeds, for example, Basmati rice is more attractive to consumers. The mutant of slr-1 for slender seeds occurs as a single recessive mutation and

results from a response phenotype to constitutive gibberellin (GA) (Ikeda et al. 2002). SLG7 is a protein located in the plasma membrane, constitutively expressed in various tissues in rice. Observation results of cellular analysis show that SLG7 produces slender grains by regulating cell divisions longitudinally, increasing cell length while transversely decreasing cell width (Zhou et al. 2015). This research aimed to utilize molecular MAS related to fragrance genes in rice in connection with a molecular breeding program.

MATERIALS AND METHODS

Plant materials

Variety of Sinar 1 as aromatic rice was used as a male gamete and the T250.7 mutant genotype as non-aromatic rice obtained from gamma-irradiated with irradiation dose of 250 Gy as female gamete. Sinar 1 variety was crossed with mutant of T250.7 with characteristic of good eating quality. The results of F1 crosses were detected using MAS in a polymerase chain reaction (PCR) to determine whether the crosses' results carried aromatic genes or not. Subsequently, selected F2 seeds with slender seed performance were planted in the F2 generation. Cross of Sinar 1 variety with the T250.7 mutant was carried out in a greenhouse at the Research Center for Radiation Process Technology, Jakarta, while molecular analysis was carried out in the DNA Laboratory of Plant Breeding at the Research Center for Radiation Process Technology, Jakarta.

Procedures

Selection of F2 plants with slender seed appearance

The selected F2 seeds with slender seeds performance germinated at the plant's nursery house for 21 days. The seedling was transferred to the experimental field, in which one seedling was laid for each hole. The ID number of each seedling started from 1.01 to 1.50 for TS1, whereas TS2 with the ID numbers starting from 2.01 to 2.50 and TS3 to TS7 was similar to those of TS1 and TS2. Approximately, 30 out of 330 plants from F2 seeds were randomly selected for aromatic genes using molecular MAS.

Selection of F3 to F5 Plant generation

In the selection for the F3 to F5 plant generation for aromatic genes, a similar method using molecular markers was applied. About 330 plants in each generation were planted in the rice fields, and 50 plants were selected randomly for DNA isolation and identification of aromatic genes using molecular markers.

DNA isolation

DNA isolation method was used for F1 to F5 plants using cetyltrimethylammonium bromide (CTAB) methods described by Doyle and Doyle (1990) using a small quantity of leaf blade with a slight modification of CTAB buffer (Ishak 2016). An approximately 100 mg of a 1-month-old rice leaf sample was cut into small pieces, and then placed in an Eppendorf tube (2 mL). Later, an iron ball (3 mm in diameter) was inserted into the tube. The leaves were crushed into fine powder in a crusher instrument for three cycles. Into the tube was added 0.5 mL of CTAB buffer solution consisting of CTAB (2%), NaCl (1.4 M), EDTA (10 mM), and Tris.HCl pH 8.0. The leaf powder in the Eppendorf tube was heated for 1 hour at 55°C. Subsequently, a mixture of chloroform containing isoamyl alcohol (24:1) solution was added. The solution mixture in the tube was shaken until it was thoroughly mixed. After mixing the mixture, it was centrifuged at 12,000 rpm for 8 minutes. The discarded supernatant was transferred to a new Eppendorf tube and two volumes of isopropyl alcohol added and stored in a freezer at -20°C for 2 hours. By this time, the DNA had settled to the bottom of the tube. Later, centrifugation was carried out at 13,000 rpm for 8 minutes. The discarded supernatant and the tube were dried at room temperature for 30 minutes. After the DNA tube was dry, it was then diluted with RNAase-free water. The DNA concentration was calculated using a nanodrop instrument.

Polymerase Chain Reaction (PCR)

PCR was carried out in a 200 ul of PCR tube consisting of 1.25 unit taq DNA polymerase, 200 µM DNTP, 2.5 mM $MgCl_2$, 200 nM each of primer, and 50 nM DNA template. Aromatic rice genes from F1 to F5 plants with slender seeds were selected by MAS according to the method proposed by Bradbury et al. (2005). The DNA samples from F1 to F5 plants were used for PCR with a specific primer for rice fragrance. The primer design originates from Bradbury et al. (2005) as follows: EAP (5′-AGTGCTTTACAAAGTCCCGC-3′), ESP (5′-TTGTTTGGAGCTTGCTGATG-3′), INSP (5′-TGGTAAA AAGATTATGGCTTCA-3′), and IFAP (5′-CATAGGAGCAGCTGA AATATATACC-3′). While PCR running steps were predenaturing step at 94°C for 2 minutes; followed by 40 cycles of denaturing step at 94°C for 10 seconds, annealing step at 55°C for 10 seconds, extension step at 72°C for 10 seconds; final extension step at 72°C for 7 minutes, and hold at 24°C.

Electrophoresis

Agarose gel electrophoresis of DNA samples after PCR was carried out using 1.2% agarose in TAE buffer. Later, 10 µL of diamond dye was added for every 100 mL of agarose gel. The electrical current was set at around 40 mA with a running time of 30 minutes. Visualization of DNA fragments was carried out using UV at wv of 320 nm in a UVP 310 Gel-doc imaging system with a camera 2.0 megapixels connected to the computer.

Data analysis

Chi-square test was applied at F2 and F3 plants from data derived from PCR products. Segregation detected from homo-heterozygote of the aromatic gene to non-aromatic using the formula as follows:

$$X^2 = \sum \frac{(Observed\ value - Expected\ value)^2}{(Expected\ value)}$$

Chi-square Table was 3.84 for df = 1, with a siginificant level P=0.05, when Chi-square does not exceed the critical value null hypothesis will be accepted, and the data follow hypothetical pattern.

RESULTS AND DISCUSSION

Molecular identification of aromatic gene at F1 Plants

Fragrance rice of Sinar 1 variety was a mutant variety from Indonesia and it was released as a new variety in 2020 by the National Nuclear Energy Agency (BATAN) through Agriculture Ministry decree of the Republic of Indonesia No. 850/HK.540/C/06/2020. Sinar 1 variety was used as a male gamete crossed with rice mutant T250.7 (non-aromatic) as a female gamete, the results of which was called TS. DNA marker was used in a PCR to detect whether the offspring carried the aromatic gene or not. Seven out of 43 seeds were randomly selected for seedling growth and detection of the aromatic gene in each plant. The results of molecular identification showed that six out of seven F1 plants carried the aromatic gene in heterozygotes shown at lanes 1-7, lane 8 duplicated from lane 7, and lanes 9-11 was non-aromatic rice. Each plant carrying the aromatic gene was assigned an ID number, such as TS1 to TS7. The DNA fragment size after electrophoresis was calculated for the aromatic gene as 257 bp (Figure 1). These seven F1 plants were grown until harvested in plastic pots with diameter of 30 cm and 20 cm in height.

A previous study of Sinar 1 variety showed that the second mutation occurred in the BADH2 gene with an early stop codon (Ishak 2016). The aroma of Sinar 1 variety exceeded that of other aromatic rice, such as the sintanur or Sinar 2 varieties due to the repressor gene (BADH2) losing its function. Thus, in 2 acetyl pyrroline synthesis was not blocked. The DNA fragment size of 257 bp was specific to the aromatic rice sub-species indica, but was not in the javanica rice sub-species (data was not shown). According to Bradbury et al. (2005), this gene marker can differentiate between fragrant and non-fragrant rice varieties and identified homozygous fragrant, homozygous non-fragrant, and heterozygous non-fragrant individuals in a population. Chi and Ngon (2016) reported that a sensory test using 1.7% KOH can be applied to detect aroma and non-aroma in rice. Our experiences showed that this DNA marker did not function to distinguish between aromatic and non-aromatic javanica subspecies, such as pandan wangi and rojolele as aromatic local varieties from Indonesia. The possible biochemical pathway of synthesis of two acetyl pyrrolines differed from that of the indica subspecies. Li et al. (2020) reported that DNA markers of two functional SNP, i.e., Badh2-E2 and Badh2-E7 allelic variations, could be used for genotype and genetic improvement of rice fragrance through marker-assisted selection. Molecular breeding can be developed in agriculture immediately because some DNA makers for specific traits have already been established. According to Yeap et al. (2013), analysis of genotypic and phenotypic could detect aroma directly in F1 individual plants derived from both parents. However, molecular analysis or sensory evaluation alone could not ultimately determine aromatic condition.

The yield component of F1 plants

The results indicated that the number of tillers and grains/panicles of F1 plants were higher than their parents (Table 1). It could be due to heterosis in plant breeding. According to Birchler et al. (2010), heterosis refers to the phenomenon that progeny of diverse varieties of a species or crosses between species exhibit more biomass, growth rate, and fertility than those of both parents. Observation showed that the number of tillers of each F1 plant varied, where TS3 produced 46 tillers/pot, TS1 was 40 tillers, and T250.7 as parent had 15 tillers (Table 1). F2 seeds were selected from the parents with long grain appearance and grown for F2 plants. Distinguishing medium-sized seeds from slender seeds was rather difficult because slender seeds were controlled by a recessive gene. The number of seeds per panicle varied, ranging from 210 to 255. The highest number of seeds per panicle was obtained in the TS3 line, while the lowest one was from the TS6 line.

Table 1. Yield component of F1 plants and their parents

F1 Plants	Number of tillers	Panicle length (cm)	Grains/panicle
TS1	40	30	231
TS2	38	30	220
TS3	46	29	255
TS4	32	30	254
TS5	32	30	249
TS6	12	28	210
TS7	24	30	234
Parents			
T250.7	15	29	196
Sinar 1	19	30	187

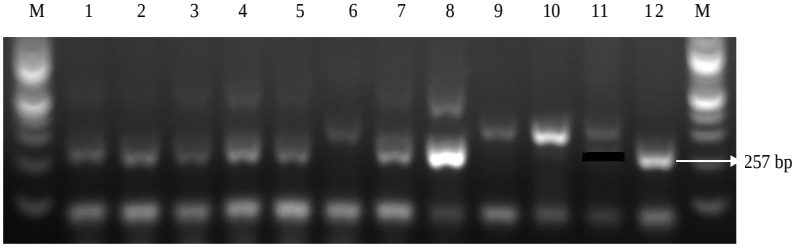

Figure 1. Marker-assisted selection of aromatic rice in F1 plants. lane 1-8: F1 plants, 9: ciherang, 10: IR64, 11: T250.7 (non-aromatic rice), 12: Sinar 1, M: DNA marker

Molecular marker-assisted selection on F2 and F3 plants

The selected F2 plants that carried the aromatic gene and produced F3 seeds which were then planted for the next generation. The results of the segregation analysis from F2 plants based on DNA-banding pattern analysis showed that 75% carried aromatic genes, both hetero and homozygous (Table 2). The leaf of F2 plants were randomly selected for DNA isolation and run for PCR. The results showed that selected samples, such as TS.221, TS.222, and TS.252, aromatic genes were not detected. The detection of fragrance in F3 plants is similar to that of aroma in F2 plants using a DNA marker. The results of analysis using MAS from 41 of F3 plant samples showed aroma gene segregation, in which 10 samples indicated the absence of a DNA fragment size of 257 bp (Table 3). Observations on F4 plants for aroma gene selection continued using MAS. The analysis results showed that four plants were still found to contain no aroma genes (Table 4). The percentage of plants without aroma genes in F4 plants was less than those of F2 and F3 plants. In contrast, the selection of aromatics without using a DNA marker will take quite a long time, which is estimated to require more than 5 years. Fragrance in rice growth can be tasted by aroma during the vegetative phase of growth. The rice aroma became strong when entering a generative phase of growth. The selection using marker-assisted selection to observe aroma in rice can be done at a month-old plant.

Table 2. The use of marker assisted-selection for rice fragrance at F2 plants

ID. numbers selected-F2 plants	Rice fragrance	DNA fragment size (bp)
137	+	257
142	+	257
149	+	257
151	+	257
221	-	-
222	-	-
227	+	257
252	-	-
239	+	257
324	-	-
327	-	-
329	-	-
3310	+	257
414	-	-
414	-	-
428	+	257
429	+	257
510	+	257
521	+	257
523	+	257
528	+	257
541	+	257
721	+	257
713	+	257
Parents		
Sinar 1	+	257
T250.7	-	-

The results showed that with MAS of the F5 plant generation, aroma homozygous plants could be obtained within two years. Based on the data from PCR and phenotypically, it showed that the F5 plant with ID numbers 761.3, 721.0, and 721.2 had a strong aroma, which was possibly due to homozygous in fragrance of rice. The selected seeds were planted to obtain F6 plants, then they were multiplied to produce core seeds. Although homozygous aroma plants were obtained, the morphological appearance of the plants was still not uniform, especially the plant height with ID number 721.A and 721.B, because the T250.7 mutant line when crossed with Sinar 1 variety was still the M3 generation. Therefore, genetic variability among individual plants caused by gamma irradiation effects was still high.

Table 3. The use of marker-assisted selection for rice fragrance at F3 plants

ID numbers selected-F2 Plants	Rice fragrance	DNA fragment size (bp)
112	+	257
114	+	257
123	+	257
134	+	257
136	+	257
141	-	-
142	+	257
143	-	-
145	-	-
153	-	-
224	-	-
254	-	-
325	+	257
333	+	257
335	+	257
331.0	+	257
322	-	-
344	-	-
415	-	-
420	+	257
422	+	257
423	+	257
432	+	257
434	+	257
514	+	257
511.0	+	257
519	+	257
527	+	257
528	+	257
529	+	257
535	+	257
539	+	257
541	+	257
547	+	257
711	-	-
714	+	257
717	+	257
721	+	257
721.0	+	257
737	-	-
761	+	257

Table 4. The use of marker-assisted selection for rice fragrance at F4 plants

ID numbers selected-F2 plants	Rice fragrance	DNA fragment size (bp)
541.1	+	257
542.2	+	257
541.3	+	257
541.4	+	257
541.5	+	257
541.6	+	257
541.7	+	257
541.8	+	257
541.9	+	257
541.10	+	257
541.11	+	257
541.12	+	257
541.13	+	257
541.14	_	-
541.15	_	-
541.16	_	-
514.17	+	257
514.18	-	-
721.1	+	257
721.2	+	257
721.3	+	257
721.4	+	257
721.5	+	257
721.6	+	257
721.7	+	257
721.8	+	257
721.9	+	257
721.10	+	257
721.11	+	257
721.12	+	257
721.13	+	257
7210.1	+	257
7210..2	+	257
7210.3	+	257
7210.4	+	257
7210.5	+	257
7210..6	+	257
7210.7	+	257
7210.8	+	257
7210.9	+	257
7210.10	+	257
7210.11	+	257
7210.12	+	257
7210.13	+	257
7210.14	+	257
7210.15	+	257
7210.16	+	257
761.1	+	257
761.2	+	257
761.3	+	257
761.4	+	257
761.5	+	257
761.6	+	257
761.7	+	257
761.8	+	257
761.9	+	257
761.10	+	257
Parents		
Sinar 1	+	257
T250.7	-	-

Segregation analysis of aromatic gene

Segregation analysis of aromatic gene with the ratio of 3:1 was conducted from results of PCR products using MAS, because this marker was dominant and it could detect hetero-homozygote of the aromatic gene to non-aromatic. A chi-square test was applied at F2 and F3 plants based on the data of PCR products (Tables 2 and 3) from homo-heterozygote of the aromatic to non-aromatic genes. Chi-square (X^2) value calculated for F2 was 0.19, while Chi-square Table=3.84, with a probability level at 0.05. Based on chi-square test, it indicated that the hypothesis of 3:1 for PCR products at F2, hetero-homozygotes aromatic: non-aromatic, was accepted. Similarly, the calculated value for F3 from PCR products was chi-square Table=3.84>0.091. The chi-square test results for the segregation of aromatic genes toward non-aromatic genes showed a ratio of 3:1 at F3 plants. The analysis of the PCR product after electrophoresis showed that the primers used gave the results of either the presence or absence of DNA fragments associated with aromatic genes. In plants that did not have aromatic genes, which DNA fragment with size of at 257 bp was not detected.

The character of slender seed or aroma in rice are controlled by recessive genes, based on Mendel's law, a crossing scheme between aromatic rice had medium seed size with non-aromatic rice with slender seeds has been calculated. The calculation showed that the aromatic homozygous genotype had probability slender seed phenotype in F2 plants around 6.2%. While aromatic homozygous genotype with medium seeds performance in F2 around 18.75%.

Marker-assisted selection in F4 to F5 plants

Using MAS in F4 plants for aromatic gene selection, it showed that most of the samples (90%) were positive for aromatic genes (Table 4). Individual plants were taken randomly from each of the previously selected numbers carrying aromatic genes with a DNA fragment size of 257 bp. Based on the results of PCR products identification from the 58 samples taken, onlyt 4 samples were non-aromatic (Table 4). Samples in the next successive generations were taken randomly from samples carrying aromatic genes at F4 plants. Four out of 18 samples from ID 541 showed non-aromatic, while ID 541.(1-12) detected aromatic (Figure 2), while lanes 13 and 14 were non-aromatic rice. Molecular identification of ID 721 at F5 plants showed homozygous condition (Figure 3). Leaf samples for DNA extraction were taken from plants originating from the same panicle.

The results of molecular analysis on F5 plants with ID number 7210 have shown that the aromatic gene is already homozygous (Figure 4). DNA samples for running PCR were taken randomly from rice plants, namely 10 plants out of 54 plants from the same panicle. The results of the DNA fragments separation by electrophoresis showed that all the samples contained specific DNA fragments of 257 bp for aromatic rice. Whereas Ciherang and T250.7 (lane 11-12) was non-aromatic rice did not show fragment size of 257 bp. Observation of plant height and harvest age showed that ID 721 and ID 761 plants were homozygous, because plant height was evenly distributed among the plants, with

an average harvest age of 90-95 after planting. Application of Molecular MAS in breeding program has considerably shortened the time for new crop varieties to be brought to the market (Hasan et al. 2021).

Seeds morphology

Seeds morphology at F5 generation of Sinar 1 variety was medium in size with the length/wide ratio was 2.83. Meanwhile, F5 seeds from ID.721 had a ratio of 4.1. According to Widiastuti et al. (2020), seeds with a ratio >3.1 are included as slender seeds, while a ratio <3 is classified medium. Palea and lemma color of Sinar1variety seeds was straw yellow which was more intense when compared to ID 721 genotype (Figure 5).

Seeds width of Sinar 1 variety ranged from 3.1 to 3.4 mm, while those of ID.721 ranged from 2.3 to 2.6 mm. The seeds length ranged from 8.3 to 9.5 mm and from 9.7 to 10.7 mm for cv. Sinar 1 and ID. 721, respectively. Seeds with an L/W ratio of less than three are called medium size (Widiastuti et al. 2020). Sun et al. (2016) reported that grain shape, panicle length, and seed shattering, play important roles in grain yield and harvest. Aromatic rice with slender seeds from crossing of Sinar 1 with T.250.7 mutant had probabilities around 6.25%. Besides, T.250.7 mutant has yet to be established because this mutant still has M3 plant generation when crossed with Sinar 1. Therefore, genetic segregation in progeny F2 and F3 plants differed from the parents.

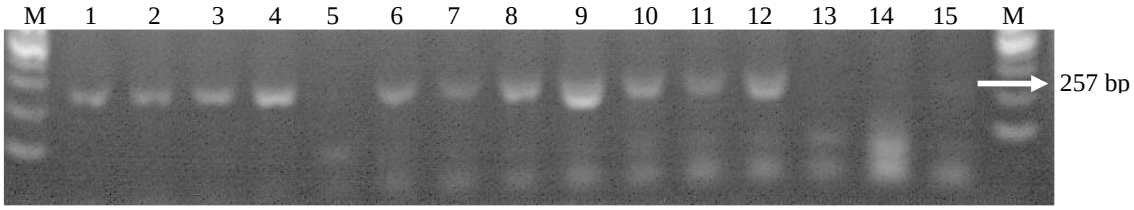

Figure 2. Identification of aromatics gene at F4 plants derived from number: lane 1-12: 541.1 to 541.12, 13: ciherang, 14: T250.7, 15: Sinar 1 (aromatic rice), M: DNA marker

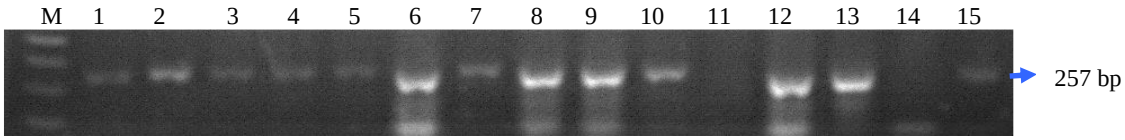

Figure 3. DNA fragment pattern from marker-assisted selection. lane 1-13: ID. 721, 14: T250.7, 15: Sinar 1, M: DNA marker

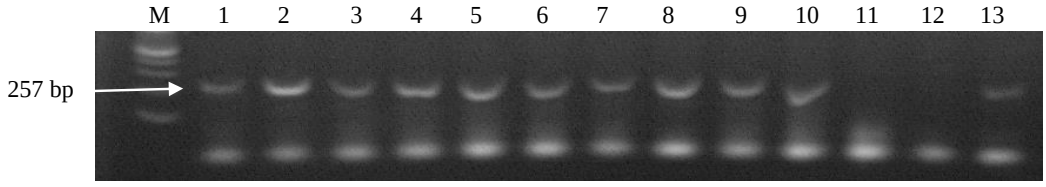

Figure 4. DNA fragment pattern from marker-assisted selection. lane 1-10: ID.7210 (F5 plants), 11: ciherang, 12: T250.7 mutant (non-aromatic), 13: Sinar 1 (aromatic rice), M: DNA marker

Figure 5. Seeds morphology. A. var. Sinar 1; B. ID.721 at F5 seeds generations

In conclusion, the use of MAS connected with molecular breeding program for fragrance rice can accelerate to obtain new lines had aroma with slender seeds. The selection of aromatic rice with slender seeds only takes two years with homozygous plant height and harvested age. It is proposed that it is necessary to develop molecular breeding in the future so that research costs are low, but require high quality human resources.

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